HEPATITIS B VIRUS VECTORS FOR GENE THERAPY

FIELD OF THE INVENTION

The present invention relates to recombinant hepatitis B viral vectors useful for the expression of heterologous genes in liver cells. The invention also provides methods for the production of novel recombinant hepatitis B viruses. Liver-specific targeting ability of these HBV vectors extend its use for *in vivo* gene therapy protocol as well as for *ex vivo* therapy protocol. The recombinant viruses produced by this invention can deliver therapeutic genes specifically to liver cells either *in vivo* therapy protocol or *ex vivo* therapy protocol. This vector can be used not only to treat liver diseases but also genetic diseases.

BACKGROUND OF THE INVENTION

Gene therapy is considered as a new healer of modern medicine since genome sequencing is nearly completed (Anderson, 1992). Numerous methods for gene therapy have been developed in recent years (Mulligan, 1993). Gene therapy vectors used in current clinical trials can be divided into two groups: viral vectors such as retroviruses, adenoviruses or adeno-associated viruses (AAV) and nonviral vectors such as liposomes or naked DNAs (Friedmann, 1999). The most critical parameter of gene therapy is the efficiency of delivery of therapeutic genes to the recipient cells. To meet this goal, vectors need to not only specifically target recipient cells but also stably express therapeutic genes so that the therapeutic effect can be achieved. Lack of tissue-specificity and lack of long-term stable expression are serious drawbacks of current gene therapy vectors (Crystal, 1995).

To obtain efficient delivery of transgenes to target cells, viral vectors are frequently employed for gene therapy protocols. In particular, vectors that are used most often are those derived from retroviruses, adenoviruses or adeno-associated viruses (Crystal, 1995). These viral vectors are nonpathogenic and are designed to be replication-incompetent in recipient cells.

Most attempts to use viral vectors for gene therapy have relied on either retrovirus vectors or adenovirus vectors. Retroviral vectors are capable of maintaining stable gene expression because of their ability to integrate into the cellular genome. However, the disadvantages of retroviral vectors are becoming increasingly clear, including their tropism for dividing cells only, the possibility of insertional

mutagenesis upon integration into the cell genome, decreased expression of the transgene over time and the possibility of generation of replication-competent retroviruses. On the other hand adenoviruses can infect nondividing cells, but can induce only transient expression of therapeutic genes. Further, repetitive administration of adenoviral vector to obtain long-term expression frequently induces severe inflammation (Yang et al., 1995). Evidently, these viral vectors need significant improvement before clinical use.

Although these viral vectors are most frequently used, they have a few unacceptable drawbacks. To improve the lack of tissue specificity, targeted viral vectors have been studied in laboratories (Douglas et al., 1999). However, it is not clear whether targeted viral vectors can be clinically used in the near future.

Regarding liver-directed gene therapy, the protocol for these viral vectors are by and large limited to *ex vivo* therapy, since these vectors lack tissue-specificity (i. e., hepatocyte-specificity). *Ex vivo* liver-directed therapy involves the surgical removal of liver cells, transduction of the liver cells *in vitro* (e. g., infection of the explanted cells with recombinant viral vectors) followed by injection of the genetically modified liver cells into the liver or spleen of the patient. A serious drawback for *ex vivo* liver- directed gene therapy is the fact that hepatocytes (i. e., liver cells) cannot be maintained and expanded in culture. Besides the technical difficulties and complexities, costs involved in each protocol are evidently astronomical.

Ideally, liver-directed gene therapy would be achieved by *in vivo* transfer of vectors which specifically target hepatocytes. Vectors derived from hepatotropic viruses, such as hepatitis B viruses (HBV), can be administered via circulation and target hepatocytes using the same receptor as the wild-type virus. However, the hepatitis B viruses have not been explored as a gene therapy vector due to lack of information on *cis*-acting elements essential for HBV genome replication.

Hepatitis B virus (HBV) is the prototype of the hepadnaviridae, a family of a small enveloped DNA virus with pronounced host and tissue specificity (Ganem, 1996). Hepadnaviruses have been found in mammals, e.g., human (HBV), woodchuck (WHV) and ground squirrels (GSHV), as well as in birds, e. g., Pekins ducks (DHBV) and grey herons (HHBV).

One of the bottlenecks in developing an HBV-derived gene therapy vector was a lack of information on *cis*-acting elements that are essential for viral genome replication. Thus, it is prerequisite to map *cis*-acting elements across the entire HBV genome.

SUMMARY OF THE INVENTION

The present invention comprises a novel hepatitis B virus vector and methods for making and using such vectors in liver-targeting gene therapy. The recombinant hepatitis B virus particles will specifically target hepatocytes of liver tissue. It is thought that the HBV vector will be particularly useful in gene transfer to liver tissue. Further, it is contemplated that the tissue specificity of the HBV vector will enable the HBV vector to be suitable even for *in vivo* therapy as well as *ex vivo* therapy. These novel HBV vectors may be used to deliver genes to liver *in vivo* by a variety of means including infection via circulation or direct injection of DNA into liver tissue.

The tropism of hepadnaviruses for hepatocytes has particular relevance to the use of HBV in gene therapy for diseases, which are caused by lack of gene expression in liver tissue. These diseases include numerous metabolic diseases, such as hemophilia lacking factor VIII or IV expression in liver. In addition, the HBV vector will be very useful to treat patients with chronic HBV infection. Since most hepatocytes of these chronic patients are equipped with packaging function (i. e., core, polymerase, and surface antigen expression), administration of the vector DNA could lead to packaging of the recombinant HBV particles, which could then infect neighboring hepatocytes. Thus, the vector DNA encoding various antiviral functions could induce therapeutic benefit. The vector DNA could be administered via direct intrahepatic injection or via circulation.

The invention provides information on two novel *cis*-acting elements of the hepatitis B virus genome that are essential for viral genome replication: α element and β element. The invention also provides a nucleotide sequence of the α element and β element.

The present invention is illustrated using recombinant HBV genome; however, the invention contemplates the use of other hepadnaviruses, including but not limited to woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV), and duck hepatitis B virus (DHBV). The art is well aware that the genomic organization of these hepadnaviruses is similar and that the teachings of the present invention can be translated to other hepadnaviruses.

In one embodiment of the present invention, the HBV vectors retain all *cis*-acting elements essential for viral genome replication. However, the present invention does not limit the position of the two novel *cis*-acting elements (i. e., α element and β

element) at the indicated position on the map. In one embodiment, it is contemplated that in order to accomodate a larger insertion without exceeding the packaging size limit, the position of novel *cis*-acting elements could be changed without compromising vector function.

The present invention also delineates methods for the encapsidation of a recombinant hepatitis B virus genome, comprising the steps of providing: i) a recombinant HBV vector encoding at least one heterologous gene sequence inserted; ii) a helper plasmid capable of providing *in trans* hepatitis B virus gene products to complement the HBV vector for encapsidation and viral genome replication; and iii) introducing the recombinant HBV vector plasmid and a helper plasmid into the liver cell under conditions such that the recombinant HBV genome is encapsidated into the viral particles. It is contemplated that the liver cells of the present invention be selected from the group consisting of human liver cells including HepG2 cells, Huh7 cells, Chang liver cells, and rodent cells.

In one embodiment of the method, a heterologous gene sequences is inserted between the α element and DR2 of the prototype HBV vector (FIG. 10). In another embodiment of the method, it is contemplated that a heterologous gene sequences is inserted between 5' epsilon and the α element of the prototype HBV vector (FIG. 8).

A few attempts were made to generate recombinant HBV viruses, in which a subset of the HBV genome was substituted by heterologous genes (Chiang et al., 1992; Chaisomchit et al., 1997; Protzer et al., 1999). The present invention significantly differs from reported US patent number 5,981,274 as follows (Chaisomchit, et al., 1997). First of all, a heterologous sequence was inserted into the spacer (or tether) domain of the HBV polymerase ORF as a fusion protein in the patent above. Contrary to this, the present invention indicates that this insertion site overlaps with the α element found to be essential for the viral genome replication in this invention. Thus, the 50-fold reduction of the viral genome replication as indicated in the patent is a consequence of disruption of the α element in the vector. Further, the size of insert (267 bp or 374 bp) and its expression as a fusion protein limits its use as a vector. In conclusion, the recombinant HBV vector claimed in the patent above is defective or not capable of packaging a recombinant HBV genome encoding a heterologous gene sequence.

In addition, it has been claimed that two HBV mutants, in which a part of the HBV genome was substituted by the 0.7 K bp luciferase gene fragment, could produce

virion particles in culture medium (Chiang et al., 1992). Further, the first successful production of recombinant hepadnaviruses (i. e., DHBV and HBV) encoding either GFP or interferon alpha were recently reported (Protzer et al., 1999). In both of these studies, without knowing the *cis*-elements essential for genome replication, they succeed in making recombinant hepadnaviruses when they substituted S ORF of DHBV or HBV with GFP or interferon-alpha in their recombinant vectors. The present invention significantly differs from the published reports in that this invention completely mapped *cis*-elements essential for viral genome replication. Based on the mapping data, this invention provides the prototype recombinant HBV vector in which heterologous sequences can be inserted into two different sites with an insert size of up to 0.90 K bp or 1.7 K bp, respectively.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. A schematic representation of the pregenomic RNA of hepatitis B virus. *cis*-elements are indicated: DR1 (direct repeat 1), DR2 (direct repeat 2), epsilon (encapsidation signal), r (repeat) element, PRE (posttranscriptional RNA processing element). Four open reading frames of HBV are represented as arrowed open boxes: precore region (nt. 1816-1902), core region (nt. 1903-2454); pre-S1 region (nt. 2850-3173), pre-S2 region (nt. 3174-156), S region (nt. 157-837); P region (nt. 2310-1625), X region (nt. 1376-1840).

FIG. 2. Life cycle of hepadnaviruses.

FIG. 3. Model for the synthesis of hepadnaviral DNA through reverse transcription of the pregenomic RNA as a template for the viral polymerase. (see text for explanation).

FIG. 4. A schematic representation of gene therapy procedure using the hepatitis B virus vector to deliver a heterologous gene (e. g., GFP) to liver cells. A recombinant HBV vector DNA encoding GFP gene is transfected into a packaging cell line that expresses viral proteins necessary for packaging the recombinant HBV genome. The produced recombinant HBV particles then infect hepatocyctes. Upon the entry of the HBV particles into cells, the viral DNA is repaired to CCC (covalently closed circular) form DNA in the nucleus and induces the expression of

GFP, as the wild-type HBV does. The packaging cell line can be replaced by a helper plasmid that provides core and the viral polymerase.

FIG. 5a. A schematic representation of the subcloning procedure of R015 plasmid.

FIG. 5b. Map of R015 plasmid. Nucleotide sequence of R015 plasmid is attached in SEQ ID 3.

FIG. 6. Map of a series of small deletion mutants. The sequence deleted in each mutant was indicated by a solid line. The plasmid names are indicated by prefix R followed by numbers. The nucleotide position and the size of the deletions are indicated.

FIG. 7. An autoradiograph of Southern blot analysis of a series of deletion mutants to determine if each deletion mutant is replication-competent. RC, relaxed circular DNA; DL, double-stranded linear DNA; SS, single-stranded DNA.

FIG. 8. Map of the prototype HBV gene therapy vector. Sequence elements of the HBV vector are drawn on the pregenomic RNA. Two novel *cis*-elements; α element, βelement. Two proposed insertion sites are indicated by open boxes. Appropriately located viral promoters are indicated; core promoter and pre-S2/S promoter, respectively.

FIG. 9. A schematic representation of subcloning procedure of R711 plasmid.

FIG. 10. A schematic representation of R711 plasmid with *cis*-acting elements.

FIG. 11a. An autoradiograph of Southern blot analysis of R711 plasmid. HBV probe was used to detect the viral replication-intermedaites. RC, relaxed circular DNA; DL, double-stranded linear DNA; SS, single-stranded DNA.

FIG. 11b. An autoradiograph of Southern blot analysis of R711 plasmid. GFP probe was used to detect the viral replication-intermediates. RC, relaxed circular

DNA; DL, double-stranded linear DNA; SS, single-stranded DNA.

Table 1. Summary of data obtained from a series of deletion mutants.

DETAILED DESCRIPTION OF THE INVENTION

A. DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

As used herein, the term "cis" is used in reference to the presence of genes on the same chromosome.

The term "trans-acting' is used in reference to the controlling effect of a regulatory gene on a gene present on a different chromosome.

As used herein, the term "in trans" is used in reference to indicate the complementation effect of a gene product on a gene present on a different chromosome.

The term "cis-acting" is used in reference to the controlling effect of a regulatory gene on a gene present on the same chromosome.

Nucleotide sequences of HBV genome were numbered according to Galibert et al. (Galibert et al., 1979), unless otherwise indicated. In this numbering system, the 5'-end of the pregenomic RNA is at nt. 1820 (Nassal et al., 1990). On the other hand, nucleotide sequences of the plasmids included in SEQ were numbered from the 5'-end of the pregenomic RNA.

B. OVERVIEW

1. Hepatitis B Viruses

Hepatitis B virus (HBV), the causative agent of chronic hepatitis in man, is the prototype member of the hepadnaviridae (Ganem, 1996). Related members of the hepadnavirus family include woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV), and duck hepatitis B virus (DHBV). HBV genome is a circular DNA of only 3.2 K bp in length. The viral genome is a partially duplex

circular DNA, possessing a single-stranded gap region in plus-strand DNA. Although HBV has a DNA genome, it replicates through reverse transcription of an RNA intermediate, the pregenomic RNA (pgRNA), within the subviral core particle. There are four major open reading frames (ORFs), all encoded in same strand (FIG. 1). Inspection of the sequence led to the recognition of conserved repeat elements that play important roles in the genome replication. These direct repeats (denoted DR1 and DR2) are located near the 5' end of the minus and plus DNA strands (FIG. 1).

The hepadnaviral life cycle is outlined in FIG. 2 (Ganem et al., 1987). Hepadnaviruses are thought to enter the hepatocytes through receptor-mediated endocytosis. Upon entry, a partial duplex genome is repaired to a covalently closed circular DNA (CCC), which is the template for transcription. Four viral transcripts are synthesized and transported to cytoplasm. The 3.5 K bp RNA, also called pregenomic RNA, serves as a template for reverse transcription as well as for translation of the core (C) and polymerase (P).

II. Reverse transcription

Despite of the general similarities to retroviruses, many steps in its replication are distinct (Nassal et al., 1996). The first step of HBV genome replication is the encapsidation of the pregenomic RNA into core particles. Core particle assembly involves the interactions of the structural proteins, core (C) and polymerase (P) with the pregenomic RNA. Incorporation of P protein as well as the pregenomic RNA into assembling core particles is essential for viral DNA synthesis. The *cis*-acting element for encapsidation, termed ε, has been defined within 85 nucleotides (nt) near the 5' end of pgRNA, which is necessary and sufficient to direct encapsidation of heterologous RNA sequences into viral core particle (Junker-Niepmann et al., 1990; Hirsch et al., 1991). The epsilon element can fold into a stem-loop structure, which is highly conserved among hepadnaviruses.

Reverse transcription mechanism of HBV polymerase is quite complicated, as expected from its peculiar genome structure. A process described as template switching is required for the successful synthesis of a double-stranded DNA product (FIG. 3). First, minus-strand DNA synthesis is initiated near the 5'-end of its template, the pregenomic RNA (Loeb et al., 1995). The viral polymerase is both the primer and polymerase for minus-strand DNA synthesis (Wang et al., 1992). Following template switching to an acceptor site near the 3' end of the pregenomic

RNA, minus-strand DNA synthesis resumes at this position, resulting in a genome-length, minus-strand DNA. Upon completion of the synthesis of minus-strand DNA, the final RNase H cleavage product, the 18-nt RNA fragment, serves as a primer for the initiation of plus-strand DNA synthesis (Loeb et al., 1991). Upon translocation to DR2, the RNA primer is used for the initiation of plus-strand DNA synthesis. For the plus-strand DNA initiated at DR2, a third template switch, termed circularization, is required to generate a mature relaxed circular DNA.

IV. *Cis*-acting elements essential for HBV genome replication

Molecular analysis revealed that several elements play a role in the viral DNA synthesis (Nassal et al., 1996). The list of *cis*-acting elements includes: 5' epsilon, encapsidation signal (Junker-Niepmann et al., 1990; Hirsch et al., 1991); DR1 and DR2, primer acceptor sites for primer translocation step during viral DNA synthesis (Nassal et al., 1996); r (repeat) for circularization (Loeb et al., 1997); and PRE, posttranscriptional RNA processing element (Huang et al., 1995). All of these known elements are located to either side of the HBV pregenome (FIG. 1). In the case of DHBV, three additional elements, termed 3E, M and 5E, have been reported to be essential for template switching during plus-strand DNA synthesis (Havert et al., 1997). However, it is not known whether any other elements in the middle of the viral genome are essential for HBV genome replication. HBV genome has not been explored as a gene therapy vector, primarily due to the lack of information on its *cis*-acting elements.

V. Design of the prototype HBV vector

Having identified all *cis*-acting elements required for HBV genome replication, it is possible to design a gene therapy vector that can accomodate a heterologous gene sequence without compromising its ability to replicate, if trans-acting factors are provided *in trans*. Briefly, the HBV vector encodes all *cis*-acting elements that are essential for the viral genome replication, but lacks expression of the viral proteins. Nonetheless, the recombinant virus can be produced if the viral proteins (i. e., core, polymerase, surface antigens) are provided *in trans* via a helper plasmid or packaging cell lines (FIG. 4).

Several issues need to be considered for the design of a gene therapy vector including insertion site, size of insert(s), promoter to drive transgene transcription.

First of all, two insertion sites were selected; one between 5' epsilon and the α element, the other site located between the α element and DR2 (FIG. 8). These two insertion sites were selected since these sequences are dispensable for viral genome replication. Regarding insert size, fragments of up to 0.90 K bp and 1.7 K bp, respectively, can be inserted into these two insertion sites without significantly exceeding the wild-type genome size. Two endogenous viral promoters (i. e., core promoter and pre-S2/S promoter), conveniently located just upstream of these two insertion sites, are employed to drive transcription. Further, this HBV vector can be used as a bicistronic expression vector, if two insertion sites are used simultaneously (FIG. 8).

C. EXPERIMENTAL

Most of the techniques used for vector construction and cell transfection are widely practiced in the art, and most practitioners are familiar with standard resource materials describing specific conditions and procedures.

Construction of the vectors of the invention employs standard ligation and restriction techniques which are well understood in the art (see Sambrook et al., 2001).

In this experimental disclosure, the following abbreviations are applied: M (molar), mM (millimolar), ml (milliliters), μg (micrograms), mg (milligrams), PEG (polyethylene glycol), ORF (open reading frame),

The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the disclosure.

EXAMPLE 1

Construction of a replication-competent plasmid for wild-type HBV

To design a HBV gene therapy vector, it is prerequisite to map all the *cis*-acting elements that are essential for the viral genome replication. To achieve this, a replication-competent plasmid that can lead to the production of infectious HBV particles upon transfection was constructed. The fact that a heterologous promoter driven RNA transcript analogous to the pregenomic RNA can lead to the production of infectious viral particles are well understood in the art (Nassal et al., 1990). Thus, a pregenomic RNA expression plasmid was designed such that the 5'-end of the

transcripts would be identical to that of wild-type HBV. Specifically, the position of 5'-epsilon element is 30 nucleotides away from the 5'-end (Jeong et al., 2000).

The nucleotide sequence of the HBV genome was numbered starting at the unique Eco RI site of HBV ayw subtype, according to the method of Galibert et al. (Galibert et al., 1979). Nucleotide numbers (nt.) indicate the HBV sequence number, unless otherwise indicated. In this number system, the 5' end of the pregenomic RNA is at nt. 1820 (Nassal et al., 1990).

1-1. Construction of R402 plasmid (pCMV-HBV/164):

To generate a replication-competent HBV construct, the greater-than-genome-length viral genome should be inserted downstream of the promoter element to maintain terminal redundancy of the pregenomic RNA (see FIG. 1; Ganem et al., 1987). The genome of hepatitis B virus was derived from pSV2A-Neo(HBV)2 plasmid that contains a dimer of HBV ayw subtype (Shih et al., 1989). The greater-than-genome-length Fsp I(nt. 1804)-to-Xba I(nt. 1992) fragment (3354 nt) of HBV ayw subtype (Galibert et al., 1979) was inserted into Eco RV and Xba I sites in the multiple cloning site of pcDNA1/Amp plasmid (Invitrogen, U.S.A.): R402 plasmid (pCMV-HBV/164). The HBV transcript made from this plasmid (pCMV-HBV/164) has a vector-derived 134 nt at the 5' end relative to that of wild-type pregenomic RNA.

1-2. Construction of R015 plasmid (pCMV-HBV/30):

To make a RNA expression plasmid that can transcribe the HBV pregenomic RNA that is almost identical to the wild-type pregenomic RNA with respect to the position of the epsilon element, a small deletion was introduced into R402 plasmid (pCMV-HBV/164). Thus, R015 plasmid (pCMV-HBV/30) was made by removing this pcDNA1/Amp plasmid-derived 134 nucleotides by a PCR-mediated method (Jeong et al., 2000).

Briefly, a fragment was made by polymerase chain reaction using a forward primer of the sequence 5 -CCCGAGCTCTCTGGCTAACTAACTTTTTCACCTCTGCC-3 (*Sac*I site underlined) and a reverse primer of the sequence 5 -CCCAAGCTTCTATTGTTCCCAAGAATATGG-3 (nt 2839 to 2822) with R402 (pCMV-HBV/164) as a template. The resulting PCR fragment was digested by *Sac*I

and *Bsp*EI and then inserted between the *Sac*I (nt. 2894 of pcDNA1/amp) and *Bsp*EI (nt. 2331) site of R402 (pCMV-HBV/164).

1-3. Construction of R063 (pCMV- CPS) helper plasmid

Briefly, PCR was carried out using a forward primer with EcoR I site and a reverse primer with Xho I site to generate the EcoR I-to-Xho I fragment (nt. 1903-to-2454) Then, the 0.5 K bp PCR product was inserted into pcDNA3 (Invitrogen, U. S. A) via EcoR I, Xho I restriction sites to make R062 plasmid. Next, the BspE I (nt. 2331)-to-Apa I of R062 plasmid was substituted by 2.6 K bp BspE I (nt. 2331)-to-Apa I of R015 plasmid. This R063 plasmid lacking encapsidation signal, epsilon, was employed as helper plasmid to provide the viral proteins (i. e., core, polymerase, surface antigen) essential for the viral replication and assembly.

Forward primer: 5'-CATGGAATTCATGGACATCGACCCT-3
(EcoR I site underlined)
Reverse primer: 5'-CCGCTCGAGCTAACATTGAGATTCCCGAGA-3'
(Xho I site underlined)

Forward primer: 5'-CATGGAATTCATGGACATCGACCCT-3
(EcoR I site underlined)
Reverse primer: 5'-CCGCTCGAGCTAACATTGAGATTCCCGAGA-3'
(Xho I site underlined)

EXAMPLE 2

Demonstration of replication-competency of wild-type pregenomic RNA expression plasmid, R015 plasmid (pCMV-HBV/30).

2-1. Cell growth, transfection of heptoma cell lines

Human hepatoma cells, designated Huh7 cells were grown in DMEM (Gibco-BRL) supplemented with 10 % fetal bovine serum (Gibco-BRL) and 10 □ of gentamicin per mL and were split every third day. The day before transfection, cells were plated at a confluency of 75 %. On the following day, cells were washed twice with phosphate- buffered saline (PBS) and given fresh media. After 2 hours, cells were transfected with 10 □ of supercoiled plasmid DNA per 60 mm plate by the

CaPO4 coprecipitation technique.

2-2. Southern blot analysis of the viral replication-intermediate from cytoplasmic core particles.

Three days after transfection, viral DNAs were extracted from intracellular core particles by PEG precipitation as described previously (Staprans et al., 1991). Briefly, transfected cells from a 100-mm plate were lysed in lysis buffer [10 mM Tris (pH 7.5), 1 mM EDTA, 50 mM NaCl, 8 % sucrose, 0.25 % Nonidet P-40]. Nuclei were removed by centrifugation for 3 min in a microcentrifuge, and the cytoplasmic extract was adjusted to 6 mM $MgCl_2$ and digested with DNase I (50 □/□) for 30 min at 37°C. Cores were precipitated by centrifugation for 4 min after adding 4 X PNE buffer [26% PEG, 1.4 M NaCl, 25 mM EDTA], and incubating at 4 °C for 30 min.. Core particles resuspended in buffer [10 mM Tris (pH 7.5), 6 mM $MgCl_2$] were then digested with DNase I for an additional 15 min at 37° C, followed by the addition of 5 mM EDTA, 1 % SDS, and 500 □ of proteinase K per □ and were incubated for 1 h at 37 °C. Core nucleic acid was extracted twice with phenol/$CHCl_3$ (1:1) and precipitated with ethanol, then resuspended in 50 □ of TE [10 mM Tris(pH 7.5), 1 mM EDTA].

Extracted viral DNA were subjected to agarose gel electrophoresis, followed by Southern blot analysis, which are well known to those skilled in the art (*Current Protocols in Molecular Biology*, Ausubel, F. et al., eds., Wiley and Sons, New York, 1995).

EXAMPLE 3

Deletion mutants of R015 plasmid (pCMV-HBV/30).

A series of small deletion mutants were generated by standard recombinant DNA technology (Sambrook et al., 2001).

3-1. R060(pCMV-ayw Δ1910-1992) plasmid

Plasmid R059(pBS+Δ1910-1992) was made in which the Sac I-to-EcoR I(nt. 3182) fragment of HBV ayw subtype, but lacking the nt. 1910-1992 fragment (Galibert et al., 1979) was subcloned into pBluescript SK(+) plasmid (Stratagene, USA). Subsequently, the Sac I-to-EcoR I fragment of R015 was replaced by the Sac I-to-EcoR I fragment of R059 to generate R060 plasmid.

3-2. R048(pCMV-ayw Δ1884-2459) plasmid

First, plasmid R046 was made in which the Sac I-to-EcoR I(nt. 3182) fragment of HBV ayw subtype (Galibert et al., 1979) was subcloned into pCH110 (Pharmacia). Then, R047 plasmid was generated by deleting the 151 bp Xba I fragment (nt. 1992-2143). Subsequently, the Sac I-to-EcoR I fragment of R015 plasmid was replaced by the Sac I-to-EcoR I fragment of R047 to generate R048 plasmid.

3-3. R056(pCMV-ayw Δ2143-2459) plasmid

First, plasmid R049 was made in which the Sac I-to-EcoR I(nt. 3182) fragment of HBV ayw subtype (Galibert et al., 1979) was subcloned into pBluescript II KS(+) (Stratagene, U. S. A). The Sty I(nt. 1884)-to-Sty I(nt. 2459) fragment of R049 was replaced by a PCR product of Sty I(nt. 1884)-to-Xba I(nt. 2143) fragment encoding Sty I restriction site at the 5'-end of the reverse primer to make R051 plasmid. Subsequently, the Sac I-to-EcoR I fragment of R015 was replaced by the Sac I-to-EcoR I fragment of R051 plasmid to generate the R056 deletion mutant.

Forward primer: 5
-CCCGAGCTCTCTGGCTAACTAACTTTTTCACCTCTGCC-3 (*Sac* I site underlined)

Reverse primer: 5'-CCCCCCAAGGCGCTGGATCTTCCAAATT-3'
(Sty I site underlined)

3-4. R021(pCMV-ayw Δ2459-2817) plasmid

First, plasmid R407 was made in which the Sac I-to-Xho I(nt. 129) fragment of R015 plasmid was subcloned into pBlueBacHis2 plasmid (Invitrogen, U. S. A.). Then, the Sty I(nt. 2459)-to-BstE II(nt. 2817) fragment of R407 was deleted and filled in by Klenow fragment to make R018 plasmid. Subsequently, the BspE I (nt. 2331)-to-EcoR I (nt. 3182) fragment of R015 was replaced by the BspE I-to-EcoR I fragment of R018 to generate R021 deletion mutant.

3-5. R022(pCMV-ayw Δ2662-3182) plasmid

To make R022, the BstE II(nt. 2662)-to-EcoR I(nt. 3182) fragment of R015

plasmid was deleted and filled in by Klenow fragment to make R022 plasmid.

3-6. R045(pCMV-ayw Δ2839-3182) plasmid

First, plasmid R701 was made in which the BstE II(nt. 2817)-to-Sph I(nt. 1239) fragment of R015 plasmid was subcloned into pGEM-4Z plasmid (Promega, U.S.A). Then, the Bgl II(nt. 2839)-to-EcoR I(nt. 3182) fragment of R701 was deleted and filled in by Klenow fragment to make R043 plasmid. Subsequently, the BstX I (nt. 2817)-to-BstX I(nt. 620) fragment of R015 was replaced by the corresponding 642 bp BstX I fragment of R043 to generate R045 deletion mutant.

3-7. R044(pCMV-ayw Δ3052-3182) plasmid

The Bsu36 I(nt. 3052)-to-EcoR I(nt. 3182) fragment of R701 was deleted and filled in by Klenow fragment to make R042 plasmid. Subsequently, the BstX I (nt. 2817) -to-BstX I(nt. 620) fragment of R015 was replaced by the corresponding 855 bp BstX I fragment of R042 plasmid to generate R044 deletion mutant.

3-8. R023(pCMV-ayw Δ3182-129) plasmid

To make R023, the EcoR I(nt. 3182)-to-Xho I(nt. 129) fragment of R015 was deleted and filled in by Klenow fragment to make R023 plasmid.

3-9. R040(pCMV-ayw Δ129-490) plasmid

First, plasmid R037 was made in which the EcoR I(nt. 3182)-to-Sph I(nt. 1239) fragment of R015 plasmid was subcloned into pGEM-4Z plasmid (Promega, U.S.A). The Xho I(nt. 129)-to-BamH I(nt. 490) fragment of R037 plasmid was deleted and filled in by Klenow fragment to make R038 plasmid. Subsequently, the EcoR I (nt. 3182)-to-Sph I(nt. 1238) fragment of R015 plasmid was replaced by the corresponding 877 bp EcoR I-to-Sph I fragment of R038 plasmid to generate R040 deletion mutant.

3-10. R041(pCMV-ayw Δ490-827) plasmid

The BamH I(nt. 490)-to-Acc I(nt. 827) fragment of R037 was deleted and filled in by Klenow fragment to make R039 plasmid. Subsequently, the EcoR I (nt. 3182)-to-Sph I(nt. 1238) fragment of R015 plasmid was replaced by the corresponding 897 bp EcoR I-to-Sph I fragment of R039 to generate R041 deletion mutant.

3-11. R025(pCMV-ayw Δ827-1238) plasmid

First, a plasmid R050 was made in which the EcoR I(nt. 3182)-to-Apa I fragment of R015 plasmid was subcloned into pBluescript II KS(+) (Stratagene, U. S. A). The Acc I(nt. 827)-to-Sph I(nt. 1238) fragment of R050 plasmid was deleted and filled in by T4 DNA polymerase to make R008 plasmid. Subsequently, the EcoR I (nt. 3182)-to-Apa I fragment of R015 was replaced by the corresponding 1591 bp EcoR I-to-Apa I fragment of R008 to generate R025 deletion mutant.

3-12. R026(pCMV-ayw Δ1238-1374) plasmid

The Sph I(nt. 1238)-to-Nco I(nt. 1374) fragment of R050 was deleted and filled in by T4 DNA polymerase to make R009 plasmid. Subsequently, the EcoR I (nt. 3182)-to-Apa I fragment of R015 plasmid was replaced by the corresponding 1866 bp EcoR I-to-Apa I fragment of R009 plasmid to generate R026 deletion mutant.

3-13. R027(pCMV-ayw Δ1374-1419) plasmid

The Nco I(nt. 1374)-to-Aat II(nt. 1419) fragment of R050 plasmid was deleted and filled in by T4 DNA polymerase to make R012 plasmid. Subsequently, the EcoR I (nt. 3182)-to-Apa I fragment of R015 was replaced by the corresponding 1957 bp EcoR I-to-Apa I fragment of R012 to generate R027 deletion mutant.

3-14. R028(pCMV-ayw Δ1419-1804) plasmid

The Aat II(nt. 1419)-to-Fsp I(nt. 1804) fragment of R050 was deleted and filled in by T4 DNA polymerase to make R013 plasmid. Subsequently, the EcoR I (nt. 3182) -to-Apa I fragment of R015 was replaced by the corresponding 1617 bp EcoR I-to-Apa I fragment of R013 to generate R028 deletion mutant.

3-15. R053(pCMV-ayw Δ1419-1592) plasmid

The Aat II(nt. 1419)-to-Apa I fragment of R050 was replaced by the PCR product of Aat II(nt. 1592)-to-Apa I fragment encoding Aat II restriction site at the 5'-end of the forward primer to make R052 plasmid. Subsequently, the EcoR I-to-Apa I fragment of R015 was replaced by the EcoR I-to-Apa I fragment of R052 to generate R053 deletion mutant.

3-16. R035(pCMV-ayw Δ1607-1804) plasmid

The EcoR I(nt. 3182)-to-Bsa I(nt. 1607) blunted fragment of R050 plasmid was ligated with the EcoR I(nt. 3182)-to-Fsp I(nt. 1804) of R015 plasmid to make R035 plasmid.

3-17. R029(pCMV-ayw Δ1804-1884) plasmid

The Fsp I(nt. 1804)-to-Sty I(nt. 1884) fragment of R050 was deleted and filled in by Klenow polymerase to make R010 plasmid. Subsequently, the EcoR I (nt. 3182)-to-Apa I fragment of R015 was replaced by the corresponding 1922 bp EcoR I-to-Apa I fragment of R010 to generate R029 deletion mutant.

EXAMPLE 4

Analysis of *cis*-elements essential for HBV genome replication

4-1. Extraction of core-associated DNA and Southern blot analysis

Transfection, DNA extraction and Southern blots were performed as described in EXAMPLE 2-2. To complement *trans*-acting viral proteins, a helper plasmid (pCMV-CPS) that provides core protein and polymerase was cotransfected along with each deletion mutant during transfection. FIG. 11 showed a typical Southern blot result. As described above, three species of the HBV replication-intermediates can be seen in this Southern blot of core-associated viral DNA: SS (single-stranded DNA), DL (double-stranded linear DNA), and RC (relaxed circular DNA). RC form is the mature product of viral genome replication found in virions. Thus, lack of the RC form DNA in Southern blots would indicate that a *cis*-acting element essential for the viral genome synthesis is deleted in the mutants.

4-2. Analysis of *cis*-elements essential for HBV genome replication.

A series of deletion mutants was generated that encompass the entire HBV genome. Each deletion lacks a fragment of between 0.05-0.52 K bp. Southern analysis indicated that only SS DNA was detected from cells transfected by the R022 mutant (pCMV-ayw Δ2662-3182/0). To delineate the region deleted in mutant R022, two addition mutants were made: R045 (pCMV-ayw Δ2839-3182/0) and R044 (pCMV-ayw Δ3052-3182/0). As a result, RC DNA as well as SS DNA and DL DNA were detected in cells transfected by R044 mutants. Thus, a sequence deleted

in the R044 mutant is not essential for viral genome synthesis. On the other hand, only trace amount of SS DNA was detected in Southern blots of cells transfected by mutant R045. Thus, a sequence deleted in R045 is essential for the viral genome replication. Taken together, a novel *cis*-element essential for HBV genome replication, termed α element (nt 2662-3052), was identified.

In addition, mutant R028 (pCMV-ayw Δ1419-1804) lacking the DR2 element (nt. 1592-1602) was made. Southern blot analysis of cells transfected by R028 (pCMV-ayw Δ1419-1804) showed the detection of only SS DNA, but not RC DNA. This result is consistent with published reports on the role of DR2 on minus-strand DNA synthesis in DHBV (Loeb et al., 1996; Condreay et al., 1992). To delineate this region further, mutant R053 (pCMV-ayw Δ1419-1592) lacking sequence upstream of DR2 element was made. Southern blot analysis indicated detection of RC DNA from cells transfected by mutant R053. This result indicated that the sequence lacking in the R053 mutant is dispensable for HBV genome replication. To further delineate this region, R035 mutant (pCMV-ayw Δ1607-1804) lacking a sequence between DR2 and DR1 elements was made. Even SS DNA was not detected from cells transfected by the R035 mutant. Thus, the sequence between DR2 and DR1 element, termed β element, is essential for the minus-strand DNA synthesis. In addition, R029 mutant (pCMV-ayw Δ1804-1884) lacking DR1 element was made. Consistent with published data, no SS DNA was detected (Condreay et al., 1992).

In contrast, sequences deleted in some of the deletion mutants turned out to be dispensable. These mutants included R060 mutant (pCMV-ayw Δ1910-1992), R048 mutant (pCMV-ayw Δ1992-2143), R056 mutant (pCMV-ayw Δ2143-2459), R021 mutant (pCMV-ayw Δ2459-2817), R044 mutant (pCMV-ayw Δ3052-3182), R023 mutant (pCMV-ayw Δ3182-129), R040 mutant (pCMV-ayw Δ120-490), R041 (pCMV-ayw Δ490-827), R025 mutant (pCMV-ayw Δ827-1238), R026 mutant (pCMV-ayw Δ1238-1374), R027 mutant (pCMV-ayw Δ1374-1419), and R053 (pCMV-ayw Δ1419-1592).

In summary, the present invention reveals two novel *cis*-acting elements that are essential for the HBV genome replication. The complete mapping of *cis*-acting elements that are essential for HBV genome replication allowed us to design a prototype HBV vector.

EXAMPLE 5

Design of prototype HBV gene therapy vectors

The present invention reveals two novel *cis*-acting elements that are essential for the HBV genome replication. In literature, a half dozen elements have been reported to be essential in various stages of HBV genome synthesis. Among these are: 5'-epsilon for encapsidation (Hirsh et al., 1991; Junker-Niepmann et al., 1990), DR2 element (Condreay et al., 1992; Loeb et al., 1996), DR1 element (Seeger et al., 1991), r (repeat) element for circularization (Loeb et al., 1997), and PRE element for post-transcriptional RNA processing (Yen, 1998). In addition to these, the two elements identified in the invention, termed α and β, complete the mapping of *cis*-acting elements essential for HBV viral genome replication (FIG. 6).

Based on the information on these *cis*-acting elements, a prototype HBV gene therapy vector was designed (FIG. 8). The critical parameters of this gene therapy vector are the size and position of the inserts. In this prototype vector, two insertion sites were identified. First, the sequence (nt 1909-2816) between 5' epsilon and the α element could be substituted by a heterologous gene of interest. At the very least, the 0.9 K bp fragment can be substituted in this site. Since this insertion site overlaps the core open reading frame, the core promoter can then be used to drive the heterologous gene inserted. Secondly, the sequence (nt. 3052-1592) between α element and DR2 element can be substituted by a heterologous gene. A fragment up to 1.7 K bp can be substituted in this site. Similarly, this insertion site overlaps the pre-S2/S gene. Thus, the pre-S2/S promoter can be used to derive the heterologous gene inserted.

EXAMPLE 6

Construction of HBV vector containing a heterologous gene

To test feasibility of the prototype vector described in EXAMPLE 5, a HBV vector was made by the insertion of GFP (green fluorescent protein) gene at the site proposed: R711 (pCMV-HBV/GFP) (FIG. 9).

6-1. The insertion site and the promoter

The insertion site was determined by considering following points: (i) all *cis*-acting elements essential for HBV genome replication should be kept intact, (ii) the endogenous viral promoter needs to be employed to maximize the coding capacity of

the vector without exceeding the maximal packaging limit.

6-2. Insertion of GFP (green fluorescent protein) gene into the HBV vector

Insertion of the 0.7 K bp GFP fragment was facilitated by polymerase chain reaction (PCR). Restriction sites were created at the end of the PCR fragment by an appropriately designed PCR primer. First of all, R709 (pCMV-HBV/ΔPs2GFP) construct was made by substitution of Bsu36 I(nt. 3052)-to-EcoR I(nt. 3182) fragment of R015 with the 0.7 K bp Bsu36 I-to-EcoR I fragment of PCR product encoding the GFP (green fluorescent protein) gene. The primers used for PCR were:

GFPBsuFII ;

5-GTCACTCCTCAGGCCATGAGTAAAGGAGAAG -3

*Bsu*36I

GFPEcoRII ;

5-GGAATTCCTTATTTGTATAGTTCATC -3

*Eco*RI

In this subcloning process, a subset of the pre-S2/S promoter was deleted. To make up this deletion, the Bsu36 I(nt. 3052)-to-Bsu36 I(nt. 3166) fragment was inserted into R709 plasmid to create R710 (pCMV-preHBV/GFP). Subsequently, to construct R711 (pCMV-HBV/GPF), the EcoR I(nt. 3182)-to-Sph I(nt. 1238) fragment was deleted by restriction digestion. Taken together, the 1.3 K bp fragment (EcoR I-to-Sph I) of HBV genome was substituted by the 0.7 K bp GFP fragment. Thus, the size of the genome is approximately 0.6 K bp smaller than the wild-type.

EXAMPLE 7

Confirmation of replication competency of the recombinant HBV vector

Feasibility of the recombinant HBV vector was examined by testing replication competency of the HBV vector. Huh7 cells were transfected by R711 plasmid, along with a helper, pCMV-CPS. DNA extraction and Southern blots were performed as described in EXAMPLE 2-2. DNA extracted from HepG2 2.2.15 cells was included as a control (Sells et al., 1988). FIG. 10a indicated that RC DNA was detected from cells transfected by R711. Further, the amount of RC DNA and the relative amount of three species of replication-intermediate DNA was comparable to that of R015, the

wild-type HBV clone. In addition, the replication of the HBV-GFP vector was further confirmed by using GFP probe (FIG. 10b).

All publications and patent applications cited in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not to be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

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